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(54) Title: OLIGONUCLEOTIDE INHIBITING THE EXPRESSION OF StAR-BINDING PROTEIN (SBP) GENE AND
METHOD THEREFOR

(54) 発明の名称: StAR結合蛋白質(SBP)遺伝子の発現を抑制するオリゴヌクレオチド及び方法

(57) Abstract: A means of inhibiting the production of an StAR-binding protein which binds to StAR protein serving as a choles-
terol transportation promoter and thus controls the function of the StAR protein. By damaging its function, apoptosis is introduced
specifically to a cancer cell. A protein interacting with StAR protein is found out. Then an RNA fragment homologous with a gene
sequence specific to this StAR-binding protein (SBP) is synthesized and introduced into a cancer cell. As a result, it is confirmed
that the expression of the StAR-binding protein is thus inhibited and, furthermore, the appearance of an apoptotic cell is confirmed.

(57) 要約: コレステロール輸送促進因子であるStAR蛋白質と結合し、StAR蛋白質の機能を調節するStAR結合蛋白質
の産生を抑制する手段に関し、更にその機能を傷害することにより癌細胞に特異的にアポトーシスを導入する。
StAR蛋白質と相互作用する蛋白質を見出し、このStAR結合蛋白質(SBP)に特異的な遺伝子配列と相同なRNA断
片を合成し、癌細胞内に導入した。その結果、StAR結合蛋白質の発現が抑制されることを確認し、更にアポトーシ
ス細胞の出現を確認した。

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OLIGONUCLEOTIDE INHIBITING THE EXPRESSION OF StAR-BINDING
PROTEIN (SBP) GENE AND METHOD THEREFOR

Field of the Invention

- 5 The present invention relates to a means to inhibit a production of StAR binding protein, which binds to StAR protein, i.e. a cholesterol transport stimulator, and regulates the function of StAR protein, and more particularly to a means to induce apoptosis specifically in cancer cells by impairing said function.

10 Prior Art

StAR protein (acute regulatory protein) plays an important role in transporting cholesterol from mitochondrial outer membrane to inner membrane (Endocr. Rev. 17, 221-224 (1996)). It is thought that StAR protein stimulated the steroid hormone biosynthesis in cytoplasm (Recent Prg Horm Res 54, 396-94 (1999)).

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While, apoptosis is a biological process to remove degenerated or transformed cells from a living body. Apoptotic cells are generated in cancer cells by radiation or chemotherapy and have been implicated in cancer treatment; however, said treatment has low specificity to cancer cells and has not been regarded as an effective treatment.

- 20 Therefore, people thought to induce apoptosis in cancer cells by regulating the function of StAR protein, which is a transport stimulator of cholesterol, i.e. a component of cell membrane (Proc. Natl. Acad. Sci. USA 99, 6943-6948 (2002)).

- Also, the present invention used a RNA interference technique to suppress a gene
25 expression by introducing RNA fragments into cells (Nature, 391, 806-811 (1998); WO99/32619).

Problems to be solved by the Invention

- 30 The rate of cancer cell division and growth is higher than those of normal cells and the rate of intracellular metabolism is higher than that of normal cells. Therefore, the present invention focused on the metabolism of cholesterol, which is a cell membrane component,

and intend to induce apoptosis specifically in cancer cells by inhibiting the synthesis of a protein, which binds to StAR protein, i.e. a transport stimulator of cholesterol, and regulates the function of StAR protein; and by impairing cellular function.

5 Means to solve the Problems

The inventors have been studying the mechanism of stimulation of the steroid hormone biosynthesis in cytoplasm of steroid hormone-producing cells by StAR protein. During the process of the research, the inventors screened proteins, which interact with StAR protein, it was revealed that one of obtained clones contains 2.3 kb inserted gene and
10 interacts with StAR protein. The translation product of the inserted gene was found to be StAR protein binding protein (SBP, DDBJ Accession number AB112474 (80..2428), AB112475 (431..2779), SEQ ID NO: 1).

The inventors synthesized RNA fragments homologous to the nucleotide sequence of
15 the gene encoding the StAR binding protein and introduced them into cancer cells using a RNA interference technique (Nature, 391, 806-811 (1998); WO99/32619). As the results, it was confirmed that the expression of StAR was inhibited and, furthermore, apoptotic cells emerged.

20 Namely, the present invention is a sense oligoribonucleotide, an antisense oligoribonucleotide or a double stranded RNA comprised thereof, wherein each oligoribonucleotide comprises less than 23 contiguous nucleotides of the nucleotide sequence of SEQ ID NO: 1 (human StAR binding protein gene) and contains bases
187-205 or 474-494 of SEQ ID NO: 1. The oligoribonucleotide is preferably bases
25 187-205 or 474-494 of SEQ ID NO: 1.

Moreover, the present invention is a method for inhibiting expression of SBP gene in cancer cells, comprising introducing into cancer cells the sense oligoribonucleotide, the antisense oligoribonucleotide or the double stranded RNA comprised thereof as in above.

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Still furthermore, the present invention is a kit for cancer therapy comprising 1) the

sense oligoribonucleotide, the antisense oligoribonucleotide or the double stranded RNA comprised thereof as in above and 2) a means to introduce into cancer cells the sense oligoribonucleotide, the antisense oligoribonucleotide or the double stranded RNA comprised thereof.

5

Brief Description of the Drawings

Figure 1 shows the result of a pull-down assay of StAR protein and clone 4.

Figure 2 shows the SBP expression in a human tissue.

Figure 3 shows SBP expression in various cell lines. HepG2 indicates human
10 hepatoma cells; KGN indicates human granulosa tumor cells; H295R indicates human
adrenal tumor cells; MCF-7 indicates human breast cancer cells.

Figure 4 shows the effect of SBP on steroid biosynthesis. The value shows the
increase in the production of pregnenolone by co-introduction of cytochrome P450 scc
system and 0.05 μ g SBP.

15 Figure 5 shows the amount of pregnenolone produced in H295R cells treated with
siRNA-SBP-I and siRNA-SBP-II.

Figure 6 shows the amount of pregnenolone produced in KGN cells treated with
siRNA-SBP-I and siRNA-SBP-II.

Figure 7 shows the emergence of apoptotic cells by introduction of siRNA (SBPII).

20 Figure 7A shows the result of introduction of siRNA-Scramble; Figure 7B shows the result
of introduction of siRNA-SBPII; Figure 7C shows the result of the treatment of those of
Figure 7B with DNAase.

Detailed Description of the Invention

25 RNA fragments used in the present invention may be sense or antisense RNA to a target
RNA, however, said RNA are easily decomposed by RNase and are not effective enough to
suppress. Therefore, double strand RNA comprising these RNA is preferably used. The
double strand RNA is usually prepared by synthesizing a sense RNA and an antisense RNA
separately and then hybridizing these.

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The length of said RNA fragments is considered effective at 21~23 nucleotides,

however generally a fragment comprising 21 nucleotides is preferably used and, in the examples described later, a fragment comprising 21 nucleotides is actually functional.

The target cells in the present invention are human cancer cells.

5 The oligoribonucleotide "substantially identical to" a specific nucleotide sequence of SBP gene means the specific part of mRNA generated by the transcription of the gene, which corresponds to the specific nucleotide sequence of SBP gene. That is, more specifically, the ribonucleotide sequence, wherein T in the specific nucleotide sequence of
10 said SBP gene is replaced to U.

There are no restrictions on the method of introducing a RNA fragment into cells and the method includes a method by calcium phosphate, a microinjection method, a protoplast fusion method, electroporation and a method using a virus vector. A commercially
15 available transfection agents comprising liposome etc. are conveniently used.

siRNA of the present invention and the method of using this could be applied to anti-cancer agents and anti-malignant tumor agents for solid tumor: epithelial carcinoma (stomach cancer, lung cancer, hepatic carcinoma, pancreatic cancer etc.), non-solid tumor:
20 leukemia, malignant lymphoma, malignant sarcoma (osteosarcoma, fibrosarcoma etc.), hormone therapy for steroid hormone -dependent malignant tumor (breast cancer, endometrial carcinoma, prostate cancer, ovary cancer etc.), therapeutic products for estrogen-dependent benign disorder (endometriosis, uterine myoma etc.), enhancement and inhibition of sperm maturation, ovulation inducer, contraceptives, therapy of premature
25 puberty, and therapy of gender identity disorder.

The following examples illustrate this invention, however, it is not intended to limit the scope of the invention. The plasmid and cells used in the Examples are prepared or cultured by the following way.

30

Construction of a Plasmid

An EcoRI fragment, prepared by PCR using human StAR cDNA as a template, was inserted into a pACT2 vector (CLONTECH Laboratories, Inc., Palo Alto, CA), containing transcriptional factor GAL 4 activation domain (GAD), and a plasmid was constructed after removal the first 62 amino acids, which is a mitochondrial transport signal. The
5 plasmid expresses a fusion protein (GAL4-N-62-StAR) between a transcriptional factor GAL4 and StAR lacking mitochondrial transport signal (i.e. N-62-StAR).

Similarly, GAL4-StAR mutants (GAL4-R193X, GAL4-Q253X, GAL4-frameshift) were constructed.

10

Also, a plasmid (pVP16-StAR) was constructed by the insertion of an EcoRI fragment prepared from human N-62-StAR cDNA into a pVP16 vector containing an activation domain (AD) derived from VP16 protein of a simple herpes virus (CLO NTECH Laboratories, Inc.).

15

Also, a plasmid (pM-SBP) was constructed by the insertion of an EcoRI/BamHI fragment of SBP cDNA into pM vector (CLONTECH Laboratories, Inc.) containing GAL4 DNA binding domain (DNA-BD).

20

Also, a plasmid (pVP16-SBP) was constructed by the insertion of an EcoRI/BamHI fragment of SBP into pVP16 vector (CLONTECH Laboratories, Inc.). For the preparation of pM-StAR plasmid, an EcoRI fragment of N-62-StAR was cloned and was inserted into pM vector (CLONTECH Laboratories, Inc).

25

PG5luc (Promega Corp., Madison, WI) contains CAT gene or Luciferase gene as a reporter. An expression plasmid (pSBP) was obtained by the insertion of an EcoRI fragment of the whole coding region amplified by PCR from testis cDNA as a result of RACE into pTarget vector (Promega. Corp.).

30

These plasmids were transfected by the use of Qiagen Maxiprep system (Qiagen, Hilden, Germany).

Cell culture

COS-1 cells and human G2 cells are obtained from Riken Cell Bank. Human adrenal cortex tumor cells H295R were gifted from Dr. Okamoto (Osaka University). Human MCF-7 breast cancer cells were obtained from ATCC (Manassas, VA). Human granulosa tumor cells KGN was gifted from Dr. Nishi (Kyushu University).

COS-1 cells were grown on 35 mm plastic dish in DMEM medium supplemented with 10% faetal calf serum and 50 μ g/ml Gentamycin.

KGN cells were cultured in DMEM/F12 medium supplemented with 10% faetal calf serum and 50 μ g/ml Gentamycin.

H295R cells were cultured in DMEM/F12 medium containing 2% ULTROSER G (BioSeptra, Cergy-Pontoise, France) and 1% ITS Premix (Becton Dickinson and Co., Franklin Lakes, NJ).

Example 1

In this invention, the protein interacting with StAR protein was identified by the use of GAL4-based yeast two-hybrid system.

Yeast two-hybrid interaction screening was performed as follows;

pCAT2 plasmid containing human testis cDNA and a reporter gene, which expresses a fusion protein (GAL4-N-62-StAR) between a transcriptional factor GAL4 and StAR (i.e., N-62-StAR) lacking a mitochondrial transport signal, were transduced into a yeast strain CG-1945 (MAT α , ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, I12, gal4-542, gal80-538, cyh². LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, URA 3: :GAL417_{mers(x3)}-CyC1_{TATA}-lacZ) (MATCHMAKER Two-Hybrid System 2, C LONTECH). Transformants (1×10^6) were cultured in a selective synthetic medium (SD) without histidine, leucine and tryptophan at 30°C for 5 days. Whole yeast DNA was transduced into E. coli HB101 by an electroporation. The transduced E. coli cells were cultured and selected in M9 medium without leucine, and then, plasmid DNA was isolated from whole His⁺ and LacZ⁺

clones.

Nine clones were obtained. These clones were classified into 3 groups based on DNA sequencing and data based analysis. As shown in Table 1, they are α -helixed coiled-coil rod homolog (HCR, accession NM019052), rabaptin-5 (RAB5EP, accession NM 004703), and nucleobindin 2 (NUCB2, accession NM 005013). HEC and NUCB2 express LacZ phenotype. The clone 4 was selected and analyzed based on the cytoplasmic localization by the prediction of the protein encoded in these clones.

As the results, the clone 4 was found to contain 2.3 kb inserted gene encoding an estimated cytoplasmic protein, which includes 1972 nt open reading frame encoding a protein comprising 657 amino acids and 62 nt non-translated sequence at the 3' end.

Table 1

Clone	Insert size (kb)	Identity	Colony color
1	-	-	
4	2.3	HCR	positive
5	2.2	HCR	positive
11	3.8	RAB5EP	negative
28	2.6	NUCB2	positive
36	0.8	HCR	negative
44	-	-	
45	4.3	RAB5EP	negative
49	2	HCR	positive

Example 2

In this example, a plasmid expressing GAD-clone 4 fusion protein and a plasmid expressing a fusion protein between GAL4-N-62-StAR and GAL4-StAR mutant was examined for the information on the interaction of StAR protein with the clone 4 protein *in vivo*.

A yeast strain Y187 (CLONTECH Laboratories, Inc.) comprising the genotype of MAT α , ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4 Δ , met-, gal80 Δ , URA3::gal417-*invers*(x3)-CyC1_{TATA}-lacZ was used. The Y187 strain was transformed with a fusion plasmid construct between GAL4 and GAD. The transformants are cultured in a selection medium (SD) without leucine and tryptophan at 30°C for 5 days. β -galactosidase activity was determined using x-gal filter assay.

Transfection was performed by the use of two kinds of reverse combinations: one reverse combination is the fusion between the clone 4 and GAL4 and the other reverse combination is the fusion between N-62-StAR and GAD. As shown the result in Table 2, the yeast cells transfected with a hybrid vector between StAR and the clone 4 showed LacZ phenotype, however those transfected with a hybrid vector between a StAR mutant and the clone 4 did not show the phenotype. The result shows that N-62-StAR interacts with the clone 4 in yeast cells.

Table 2

DNA-BD	AD	LacZ Phenotype
GAL4	GAD	white
GAL4-N-62-StAR	GAD-Clone 4	blue
GAL4-Clone4	GAD-N-62-StAR	weak blue
GAL4-R193X	GAD-Clone 4	white
GAL4-Q258X	GAD-Clone 4	white
GAL4-Frameshift	GAD-Clone 4	white

Example 3

In this example, a pull-down assay was performed for the examination of direct interaction of StAR protein with the clone 4. The pull-down assay was performed according to the following steps;

A plasmid expression clone was prepared by the insertion of an EcoRI fragment obtained by PCR into pCI vector (Promega Corp.). Then, a translation protein was prepared *in vitro* by the use of a TNT-binding reticulocyte dissolving system (Promega Corp.) based on T7 RNA polymerase. Afterward, a plasmid expressing a

CBD-N-62-StAR fusion protein (lacking 62 amino acid end) with a His tag was prepared by the insertion of a EcoRI fragment, obtained by PCR by the use of StAR cDNA as a template, into pET38b, containing a His tag (Novagen, San Diego, CA) at the C-terminal. CBD is cellulose binding domain sequence, which is characterized by binding specifically to cellulose and fix a fusion protein to an inactive carrier such as cellulose or chitin without a chemical modification.

N-62-STAR fusion protein with His tags, bound to His binding resins (Novagen), was incubated in 250 μ l buffer solution (50 mM potassium phosphate, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 0.1 % Triton-X) together with 50 μ l translated clone labeled with ^{35}S methionine for 3 hrs. The resins were collected by a micro-centrifugator and were washed for 3 times. The washed beads were suspended in 20 μ l 2xSDS sample buffer solution, heated for 5 min and pelettized. The supernatant was applied to SDS-PAGE and then to autoradiography. Figure 1 shows the result. It is shown that the clone 4 translated protein interacts with CBD-N-62-StAR fusion protein but not with CBD. The clone 4 is referred to as StAR binding protein (SBP) (DDBJ Accession number AB112474 (80..2428), AB112475 (431..2779), SEQ ID NO: 1).

Example 4

In this example, the expression of SBP was examined by Northern blot analysis.

Northern blot was performed for each 2 μ g of poly A and RNA isolated from each tissue by the use of SBP as a probe and β actin cDNA as a positive control. The expression of SBP gene was detected in all tissues examined. Figure 2 shows that the transcription products are abundant in tissues with 2.4 kb or 3.8 kb product.

For the examination of the expression of SBP in various cell lines, RT-PCR was performed by the use of mRNA isolated from HepG2 cells (human hepatic cancer cells), KGN cells (human granulosa tumor cells), H295R cells (human adrenal tumor cells) and MCF-7 cells (human breast cancer cells).

The procedure of RT-PCR was as follows;

mRNA used was isolated from Hep G2 cells, KGN cells, H295R cells and MCF-7 cells. Complementary DNA was synthesized by the use of 150 pmol oligodT as a primer, 1 µg total RNA and 200 unit SUPERScript II Rnase H (Life Technologies, Inc./BRL, Washington, DC) at 37°C for 60 min. The reaction mixture (20 µl) containing reverse
5 transcriptase comprises 50 mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl₂, 20 mM dithiothreitol and 0.5 mM each of dATP, dCTP, dGTP and dTTP. Then, SBP was amplified by the use of synthesized oligonucleotides of SEQ ID NOs: 2 and 3 as primers. The PCR reaction mixture (50 µl) comprises 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs and 10 pmol of each primer. PCR reaction condition is 35
10 cycles, wherein each cycle comprises denaturation at 94°C for 45 sec, the second annealing at 55°C for 45 sec and extension at 72°C for 1 min.

As shown the results in Fig.3, amplified products (400 bp) were obtained from all the cell lines.

15

The above results show that SBP gene is expressed in steroid hormone-producing cells such as H395R cells and KGN cells.

Example 5

20 In this example, the effect of SBP on steroid biosynthesis was examined.

COS-1 cells was cotransfected with F2, cytochrome P450 cholesterol side chain breakage system (Dr. Walter L Miller of the University of California), pStAR (pSPORT StAR CDNA) and pSBP by the use of FuGENE 6. The cells (F2/StAR/SDP) were
25 incubated for 48 hrs after the transfection. Several culture dishes were treated with 22R-hydroxy-cholesterol during the culture of the last 24 hrs. At 48 hrs after the transfection, the medium was recovered and an immunoassay of pregnenolon was performed. The results of the assay were standardized by the concentration of serum pregnenolon, which was produced in the medium containing 22R-hydroxy-cholesterol, to
30 correct the variation of the efficiency of the transfection. Each assay was repeated for three times.

Figure 4 shows the results. The amount of pregnenolone produced by co-transfected COS-1 cells (F2/StAR/SDP) is 138% of that produced in cells (F2/StAR) transfected with F2, StAR or blank vectors.

5

Example 6

In this example, SBP expression was suppressed by interference RNA and steroid hormones were examined. Two target sequences in SBP gene was selected and the effect of siRNA was measured by RT-PCR.

10

The interference RNA assay was performed as follows;

The cultures of subconfluent (40~50% confluent) H295 cells and KGN cells were seeded to 35 mm dishes at similar cell numbers. Nineteen nucleotides double strand RNA (siRNA-SBP-1) and 21 nucleotides double strand RNA (siRNA-SBP-2) were added to said cultures and said cells were transformed to be targeted their endogeneous SBP mRNA (Dharmacon, Inc., Lafayette, Co). The double strand RNAs target 187 and 474 nucleotides downstream from the initiation codon of SBP (SEQ ID NO: 1), respectively.

The siRNAs comprise oligoribonucleotide pairs SBP-I and SBP-II. SBP-I is 5'-CGGGAUGUUUCCAGUGACAdTdT-3' (SEQ ID NO: 4) and 5'-UGUCACUGGAAACAUCCCGdTdT-3' (SEQ ID NO: 5), SBP-II is 5'-GAACUUGGAAGAGGGGAGGCAdTdT-3' (SEQ ID NO: 6) and 5'-UGCCUCCCCUCUCCAAGUUCdTdT-3' (SEQ ID No: 7). Also, a scramble oligoribonucleotide pair (siRNA-scramble), i.e. 5'-GCGCGCUUUGUAGGAUUCdTdT-3' (SEQ ID NO: 8) and 5'-CGAAUCCUACAAAGCGCGCdTdT-3' (SEQ ID NO: 9), was used as a control.

These oligonucleotides were annealed according to a Dharmacon protocol. Cells were transfected with 300 pmol each double strand using 15 μ l metafectene (Biontex Laboratories GmbH, Munich, Germany) according to the manufacturer's protocol. The dish of H395R cells was treated with 15 μ M triostane (Mochida pharmaceutical co.) for 6 hrs after the transfection for the inhibition of the enzyme activity of

3 β -hydroxysteroidhydroxylase (3 β -HSD). 3 β -HSD converts pregnenolone into progesterone. At 48 hrs after the transfection, cells were collected and a radioimmunoassay of the steroid hormone was performed. Total RNA was isolated and RT-PCR was performed by the use of primers (sense: SEQ ID NO: 10; anti sense: SEQ ID NO: 11) for GAPDH. The results are shown in Figures 5 and 6.

The amount of pregnenolone produced by H295R cells (85 \pm 5.0 ng/dish for those treated with siRNA-SBP-I, 66 \pm 8.2 ng/dish for siRNA-SBP-II), treated with siRNA-SBP-I or siRNA-SBP-II after transfected with DsRNA, was 56,5 % or 37.5 %, respectively, of that produced by H295R cells transfected with scramble siRNA, as shown in Fig. 5.

The amount of pregnenolone produced by KGN cells treated with siRNA-SBP-I or siRNA-SBP-II was 71% of 55%, respectively, of that produced by KGN cells transfected with scramble siRNA, as shown in Fig.6.

The expression level of SBP gene was decreased in both siRNA-SBP-I and siRNA-SBP-II cases. These results show that decreased expression of SBP gene by the treatment of the target sequences of SBP gene with siRNA led to the lowered production of the steroid hormone.

20

Example 7

In this example, the emergence of apoptotic cells was confirmed by the use of adrenal tumor cells, H295R cells.

25 H295R cells were cultured in DMEM/F12 medium containing 2% ULTROSER G (BioSeptra) and 1 % Premix (Becton Dickinson). The cells were subcultured at a subconfluent state (40-50%) on a 24 mm x 24 mm cover glass placed on a 35 mm plastic culture dish on the day before a gene transfer. For the suppression of the expression of SBP mRNA, the cells were transfected with siRNA-I or siRNA-II by the use of metafectene. The cultured cells were fixed by phosphate buffered saline containing 4% formaldehyde for 30 min after culturing for 24 hrs after the gene transfer. DeadEnd

30

Fluorometric TUNEL system (Promega) was used for the detection of apoptotic cells and the method was followed a protocol of the system. After two times' wash by phosphate buffered saline, the back of the cover glass was put on a slide glass and observed through a microscope. The observation was performed by the use of a fluorescence microscope
5 (Axiophot, Carl Zeiss) under the condition with 450-490 nm as an excitation wavelength and a 515-565 nm absorption filter. Those cells emitted fluorescent light is apoptotic cells with fractionated DNA. A digital camera (DXM 1200, Nikon) was loaded on a microscope and took the photographs.

The image on the photographs was processed by the use of Adobe Photoshop 5.0
10 (Adobe system). The magnification of the images is four hundredfold.

Figure 7 (B) shows the result of siRNA-SBP II transfer. Apoptosis cells are recognized entailed by siRNA-SBP-II transfer.